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Corn Fiber as a Biomass Feedstock for Production of Succinic Acid

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Abstract

The selection of an economical carbon source is a fundamental parameter to establish a successful industrial succinic acid (SA) bioprocess. In this work, corn fiber (CF), a renewable and an inexpensive source of carbohydrates, was successfully used for bioproduction of SA. Optimized liquid hot water (LHW) pretreatment followed by enzymatic hydrolysis were used to obtain corn fiber hydrolysate (CFH). Results in batch fermentation with *Actinobacillus succinogenes* showed that a control solution mimicking CFH produced 28.7 g/L of SA with a yield of 0.67 g SA/g sugars, while fermentation of CFH produced 27.8 g/L of SA with a yield of 0.61 g SA/g sugars. It was found that culture pH was a critical factor affecting SA production. In sodium acetate buffered media, SA was the major end-product with lower levels of acetic acid (AA) and formic acid (FA). When unbuffered media was used, lactic acid (LA) and ethanol were also detected.

Keywords: succinic acid, corn fiber hydrolysate, *Actinobacillus succinogenes*

1. Introduction

In recent years, some dry milling corn ethanol plants have installed Fiber Separation Technology™ (FST) (ICM, Colwich, Kansas) to improve the corn separation process and augment corn's value. Fiber is removed from the corn kernel prior to fermentation to increase fermenter capacity. The fiber removed during FST consists principally of polysaccharides and possesses a small protein content. It is primarily used to feed cattle, which is a low-value use. However, corn fiber (CF) can be converted to monomeric sugars, and these sugars can be fermented into premium products with high demand such as succinic acid (SA). One of the challenges in the utilization of lignocellulosic biomass for fuels and bioproducts is the pretreatment employed prior to saccharification to disrupt the lignocellulosic structure. The low lignin content of CF allows for a low severity pretreatment to be used, such as liquid hot water (LHW), before saccharification. LHW pretreatment can be followed by an enzymatic hydrolysis that uses low temperatures and results in minor amounts of inhibitory compounds compared to acid hydrolysis (Yoo and Pan, 2017).

SA ($C_4H_6O_4$), a dicarboxylic acid which is also known as butanedioic acid and amber acid, has a fundamental importance due to its broad application in food, pharmaceutical, agricultural, polymer, and chemical industries (Jiang et al., 2017). SA is used in the production of biodegradable polymers, resins, and coatings; also, as an acidulant, sweetener, and flavoring in the food industry (Ahn et al., 2016; Nghiem et al., 2017; Pateraki et al., 2016; Song and Lee, 2006). Conventional manufacturing of SA relies on petroleum as a feedstock. SA is produced by partial oxidation of butane, followed by catalytic hydrogenation of maleic anhydride. This approach is costly and ecologically questionable (it uses heavy metal catalysts and organic solvents and high temperatures and high pressures) (Clark, 2014).

Given the fundamental role of SA as a chemical commodity and the concerns associated with petrochemical-based production processes, alternative low-cost renewable routes from sugars have been highly sought after. SA can be produced biologically, as it is part of the central metabolism of every organism (Nghiem et al., 2017). It is theoretically possible to achieve a mass yield from both glucose and xylose of 1.12 g of SA per g of sugar through carbon dioxide (CO_2) fixation. Raw materials used in commercial SA bioproduction via fermentation consist of glycerol and sugars such as glucose. Biomass materials such as corn, sugar beets, sugarcane, and wheat are also utilized (Ahn et al., 2016; Pateraki et al., 2016). Moreover, SA production by fermentation utilizes carbon dioxide (CO_2), which could contribute to reduction of CO_2 emissions and improve the sustainability of corn ethanol plants.

Bio-based SA production includes fermentations employing wild-type bacteria, engineered bacteria, and yeast. *Actinobacillus succinogenes*, *Basfia succiniciproducens*, *Corynebacterium glutamicum*, *Escherichia coli*, *Mannheimia succiniciproducens*, *Saccharomyces cerevisiae*, and *Yarrowia lipolytica* are some of the microorganisms that have been studied and/or engineered to develop SA production (Jiang et al., 2017; Pateraki et al., 2016). Among them, *A. succinogenes* is one of the most promising wild-type bacteria strains since it is more resistant to SA and inhibitors than any other previously reported SA producer (Ferone et al., 2018; Guettler et al., 1996). It can also utilize a variety of pentoses, hexoses, and disaccharides,

including glucose, xylose, arabinose, fructose, cellobiose, maltose, and lactose (Bechthold et al., 2008; Jiang et al., 2017; Pateraki et al., 2016). In fact, various renewable nonfood biomasses, such as CF, sugarcane bagasse, corn stover, industrial hemp, and rapeseed straw, have been investigated for bio-based production of SA by *A. succinogenes* (Borges and Pereira, 2011; Chen et al., 2010, 2011; Kuglarz et al., 2018, 2016; Zheng et al., 2010). To the authors' knowledge, three studies have used CF from the wet-milling ethanol process as a raw material in the production of SA by *A. succinogenes*. In Chen et al. (2010), an SA mass yield of 72.5% based on sugars consumed and an overall yield of 45.9% based on sugars in the original CF were found. On the other hand, Chen et al. (2011) reported an SA mass yield of 67.7% from CF and spent yeast cells hydrolysate. Guettler et al. (1996) reported SA concentration up to 70.6 g/L from CF hydrolysate by a variant of *A. succinogenes* 130Z. This concentration represents a yield of 88% of SA based on the weight of glucose, xylose, and arabinose contained in the CF hydrolysate. In all three studies, CF was hydrolyzed to sugars using a dilute acid hydrolysis method, which produces more inhibitor compounds compared to the enzymatic hydrolysis method used in the present study.

One of the major challenges to establish a successful industrial SA bioprocess is the high cost associated with the sugar feedstock needed in the bioconversion process. Therefore, the present contribution regards the characterization of CF that is an inexpensive by-product from the dry-milling ethanol process, as feedstock for SA production using *A. succinogenes* 130Z. Optimized LHW pretreatment and enzymatic hydrolysis conditions were used to convert polysaccharides in CF into monomeric sugars that can be fermented by *A. succinogenes*. This investigation also reports on the effects of a pH regulator ($MgCO_3$) and sodium acetate buffer solution on SA production. CF conversion into SA could decrease costs of SA production, increase the value of CF and the profitability of corn ethanol plants, and promote sustainability in the corn industry and rural economy.

2. Materials and methods

2.1. Corn fiber preparation

Ground CF provided by E-Energy Adams, LLC (Adams, Nebraska) was analyzed as received. CF was passed through different mesh screens to determine the particle size by sieving in a Tyler Ro-Tap sieve shaker. CF was retained between 20 mesh (0.841 mm) and 60 mesh (0.250 mm). The biomass was stored at room temperature in sealed buckets for subsequent use.

2.2. Compositional analysis of corn fiber

Compositional analysis of CF was carried out from two independent samples. Starch content testing was performed in both unextracted and extractive-free samples. Total extractives, oil, ash, moisture, and protein content were analyzed on unextracted samples. Measurements of polysaccharides and lignin were determined in both extractive-free and protein-free samples (see details following).

The moisture content of CF (%) was measured with a Metter Toledo HE53 moisture analyzer. Ash content was determined according to the NREL/TP-510-42622 method (Sluiter et al., 2008a). Starch content was performed using a Megazyme total analysis kit

(K-TSHK, Megazyme Ltd., Bray, Ireland). Protein extraction was carried out according to a previous method with some modifications (Evangelista et al., 2006). The defatted CF was extracted with a mixture of 55% 1 M NaOH and 45% ethanol at 55–60°C for 2 h (15 mL of mixture/g CF). Then, CF solids were separated from the mixture and washed with 70% ethanol for 1 h at 50–60°C. Next, CF solids were washed with water for 20 min and neutralized to pH 7. Finally, CF solids were dried in an oven for 3 days at 40°C. The dried CF and the original CF samples were then analyzed for protein content. Protein analysis was performed using a LECO FP-528 nitrogen/protein analyzer instrument (LECO, St. Joseph, Michigan, USA).

Extractives, including oil, were determined quantitatively in accordance with the NREL/TP-510-42619 method with some modifications (Sluiter et al., 2008b). Sequential extraction was carried out in an ASE apparatus (Thermo Scientific™ Dionex™ ASE 350 Accelerated Solvent Extractor system). Hexane was first used to extract lipophilic compounds. Then, HPLC grade water and 190-proof USP grade ethanol were used to remove water and ethanol soluble extractives, respectively. The collected solutions were loaded into a Genevac Rocket™ Evaporator system (Genevac SP scientific, Warminster, Pennsylvania, USA) and an appropriate evaporation method was run according to the solvent present in the mixture. Polysaccharides and lignin content were performed according to the NREL/TP-510-42618 method (Sluiter et al., 2012). Both extractive samples and protein-free materials were used for these analyses to evaluate the influence of protein on sugars and lignin determination.

2.3. CF pretreatment

LHW pretreatment followed by an enzymatic hydrolysis was used in this study. The pretreatment process was first optimized to maximize sugar yields from CF. Preliminary experiments were performed to compare LHW pretreated CF samples. CF was pretreated at 160, 180, and 200°C for 10, 20, and 30 min at 15% solids loading for a total of nine pretreatment combinations. LHW pretreatment was conducted by placing a 15% solids mixture containing 75 g CF (dry basis) and 425 g water into a 1 L bench top pressure reactor (Parr Reactor Model 4848, Parr Instrument Co., Moline, Illinois, USA). The mixture was agitated at 300 rpm, heated to the selected temperature, and held at the selected temperature for the desired time. At the end of the process, solids were separated from the liquid fractions of the mixture under vacuum filtration using coffee filter paper. Pretreated solids and prehydrolysate were stored at 4°C for the subsequent hydrolysis step. Compositions of pretreated solids and prehydrolysate (liquid remaining after pretreatment) were determined and levels of hydroxymethylfurfural (HMF) and furfural in prehydrolysate were studied. Pretreated solids were subjected to enzymatic hydrolysis at 5% solids loading. Pretreated biomass and prehydrolysate were autoclaved at 121°C for 25 min prior to being subjected to enzymatic hydrolysis.

2.4. Enzymatic hydrolysis of pretreated CF

Water or prehydrolysate, citrate buffer (pH 4.8 and 50 mM) or acetate buffer (pH 5.0 and 50 mM), cellulase (Ctec2, Novozymes, Franklinton, North Carolina, USA) in the ratio of 20 FPU/g glucan (Filter Paper Units enzyme/g glucan), and pretreated CF solids were added

to 250 mL flasks and incubated in a shaker at 50°C and 200 rpm for 72 h. Pretreated CF solids loading was 5%, 10%, or 15% depending on the experiment. Hydrolysates produced using water were labeled as CF hydrolysate from water (CFHW). Hydrolysates produced using prehydrolysate were labeled as CF hydrolysate from prehydrolysate (CFHP).

2.5. CFH preparation

CFHW and CFHP were obtained by filtering the slurry acquired after enzymatic hydrolysis under vacuum filtration using Whatman #1 filter paper (Fisher Scientific, Pittsburgh, Pennsylvania). The filtrates were sterilized by pumping them through a 0.22 μm filtration unit (Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with PES Membrane). The clear solutions containing sugars were kept at 20°C until further use for fermentation.

2.6. Microorganism and media

A. succinogenes 130Z (ATCC 55618) purchased from the American Type Culture Collection (Manassas, Virginia) was used to produce SA from CF hydrolysates. The culture in the form of freeze-dried pellets was revitalized and subcultured in tryptic soy broth media (G-Biosciences, St. Louis, Missouri) with 10 g/L glucose (Fisher Chemical, Hampton, New Hampshire) (Maharaj et al., 2014) before being preserved at –80°C in 20% glycerol in 1.5 mL culture tubes and used for inoculum preparation. Prior to fermentation, *A. succinogenes* culture was inoculated to seed medium (30.0 g tryptic soy broth/L) in anaerobic culture tubes and incubated in a shaker at 37°C, 250 rpm for 14–16 h. Then, the culture was washed with sterile 0.89% sodium chloride solution and resuspended with fermentation media. The fermentation medium for the flasks, based on Maharaj et al. (2014) with some modifications, had the following composition per L: 16.0 g yeast extract, 1.0 g NaCl, 1.36 g $NaC_2H_3O_2$, 0.20 g $MgCl_2{\cdot}6H_2O$, and 0.20 g $CaCl_2{\cdot}2H_2O$. The seed and fermentation media were autoclaved at 121°C for 20 min.

2.7. Carbon sources, inoculum, and SA production

Carbon sources included CFHW, a control mimicking the sugars in CFHW (Control 1), CFHP, and a control that mimicked the sugars in CFHP (Control 2). An additional control was used and designed as control 3 to study SA production when acetate buffer was added to control 1. SA production was carried out in sealed anaerobic 250 mL flasks with a working volume of 200 ml. Before fermentation, nitrogen was used to remove excess oxygen from the media and $MgCO_3$ at 80–100% of initial glucose concentration was added to regulate pH and supply CO_2 simultaneously. Inoculum (2.5% v/v) was added to each experimental flask with a syringe from the headspace to ensure anaerobic conditions and fermentation was performed in a shaker at 37°C and 150 rpm for 48 h. Samples were withdrawn at 6 h intervals for measuring concentrations of SA, sugars, and other organic acids and ethanol concentration. A schematic representation of the complete process of SA production from CFHW can be seen in Figure 2.

2.8. HPLC methods

Concentrations of glucose, xylose, arabinose, mannose, galactose, SA, lactic acid, formic acid, acetic acid, ethanol, and furfural were measured by high-performance liquid

chromatography equipment (UltiMate™ 3000 LC System, ThermoFisher Scientific, Rockwood, Tennessee). In this study, compounds were separated using two carbohydrate ion exchange columns (Aminex HPX-87P, 300 × 7.8 mm and Aminex HPX-87H, 7.8 × 300 mm, Bio-Rad, Hercules, California) and detected by a refractive index detector at 50°C (RI101, Shodex Scientific Co. Ltd., Shanghai, China). The Aminex HPX-87P column was maintained at 80°C, and the compounds were eluted with deionized water at a flow rate of 0.6 ml/min. The Aminex HPX-87H used 5 mM of H_2SO_4 as a mobile phase with a flow rate of 0.6 ml/min, and the column temperature was 65°C.

2.9. Calculations

Recovery of polysaccharides present in CF after pretreatment (PTR) was determined by the following equation:

$$\text{PTR} = [\%\text{PS(Pretreated CF)}*\text{mass(Pretreated CF)} + \text{SCPH}*\text{Conv}*\text{PHV (L)}] / [\%\text{PS(Raw CF)}*\text{mass(Raw CF)}] \quad (1)$$

where %PS refers to the mass % of polysaccharides on a dry basis, mass (Pretreated CF) is the dry mass in g of pretreated CF collected after pretreatment, SCPH is monomer sugar concentration in g/L in prehydrolysate, Conv is the conversion factor for conversion of monomer concentration to polysaccharide concentration (0.9 for glucose, 0.88 for xylose and arabinose), PHV is the volume in L of prehydrolysate collected after pretreatment, and mass(Raw CF) is the dry mass of CF added to the pretreatment reactor, which was 75 g in this study.

Recovery of sugars present in raw CF after both pretreatment and hydrolysis (PHYR) was determined by the following equations:

$$\text{HYY} = [\text{SCH}*\text{Conv}]/[\%\text{PS(Pretreated CF)}*\text{SoH}] \quad (2)$$

$$\text{PHYR} = \{[\text{HYY}*\%\text{PS(Pretreated CF)}*\text{mass(Dry Pretreated CF)}] + [\text{SCPH (g/L)}*\text{Conv}*\text{PHV (L)}]\} / [\%\text{PS(Raw CF)}*\text{mass(Raw CF)}] \quad (3)$$

where SCH is sugar concentration in hydrolysate in g/L, SoH is solids concentration in hydrolysis in g/L, and HYY is the yield of sugars obtained in hydrolysate.

2.10. Statistical analysis

All results are presented as mean values ± one standard deviation. When indicated, comparisons between mean values were subjected to one-way analysis of variance followed by the Tukey test and two-way analysis of variance (ANOVA). The statistical differences between different groups were analyzed at 95% confidence interval using SAS (version 9.4 TS Level 1M6, SAS Inst. Inc., Cary, North Carolina, USA).

3. Results and discussion

3.1. CF composition

Prior to the removal of water and ethanol soluble extractives, it was necessary to remove lipid components from CF. For this step, a hexane extraction was performed, followed by a water-and-ethanol extraction (Sluiter et al., 2008b). The amount of protein in CF from the FST process is considerable. The method used to determine biomass composition (Sluiter et al., 2012) uses an acid hydrolysis step in which some protein is solubilized in the hydrolysis liquid. The solubilized protein interferes with the accurate determination of soluble lignin. Therefore, it was necessary to perform a protein extraction of CF prior to sugar and lignin analysis as described in Section 2.2. In addition, starch analysis showed that the amount of starch before and after the extraction steps differed significantly (data not shown here), thus it was necessary to determine starch content in unextracted and extractives-free biomass. The amount of starch in protein-free material was also analyzed; however, no starch was detected in protein-free samples.

Because of the nature of the biomass as explained above, raw CF was analyzed for polysaccharides (glucan from cellulose and starch, xylan, galactan, arabinan, and mannan), total lignin, extractives (sum of water- and alcohol-soluble extractives), oil, ash, and protein content. CF contained on a dry basis (data reported as mean of duplicates ± one standard deviation) 21.0 ± 0.7% non-starch glucan, 4.5 ± 0.4% starch, 27.5 ± 0.3% xylan, 4.3 ± 0.2% galactan, 14.6 ± 0.4% arabinan, 0.5 ± 0.1% arabinan, 0.8 ± 0.2% lignin, 4.2 ± 0.1% oil, 0.6 ± 0.0% ash, 19.3 ± 0.4% protein, and 4.5 ± 0.1% extractives.

3.2. Optimization of pretreatment and enzymatic hydrolysis of CF

Glucan and total sugar PTRs were calculated based on the initial glucan and total polysaccharide content of corn fiber (Fig. 1A). Glucan PTR for 180°C-10 min (91.4%) was significantly greater than Glucan PTRs for all other conditions except 160°C-30 min (78.6%), which was not significantly different than 180°C-10 min. Total sugar PTRs (Fig. 1A) for pretreatment conditions of 160°C-10 min (71.0%) and 180°C-10 min (64.0%) were not significantly different from one another and were significantly greater than other pretreatment conditions. Total sugar PTR decreased as temperature and time increased. PHYRs for glucan and total sugars for various pretreatment conditions are displayed in Figure 1B. Glucan (88.2%) and total sugar (62.9%) PHYRs were greatest for 180°C-10 min. The combination of high sugar yields after pretreatment and high glucose hydrolysis lead to 180°C-10 min being the optimal pretreatment condition for producing sugars for subsequent fermentation. Dien et al. (2006) reported > 95% glucose from corn fiber pretreated at 160°C, 20 min at low biomass loading (2% w/w solids). In this study, comparable glucose yields were obtained from corn fiber pretreated at a greater solids loading (15%). Based on high glucan and xylan recoveries after pretreatment and enzymatic hydrolysis of pretreated solids, pretreatment condition of 180°C, 10 min was chosen as the optimum condition for further experiments. CF was pretreated using 15% and 20% solids loadings at the pretreatment condition of 180°C, 10 min. Glucan and total sugar PHYRs were greater for CF pretreated at 15% solids than 20% solids; thus, a 15% solids loading was used for further experiments (Fig. 1C).

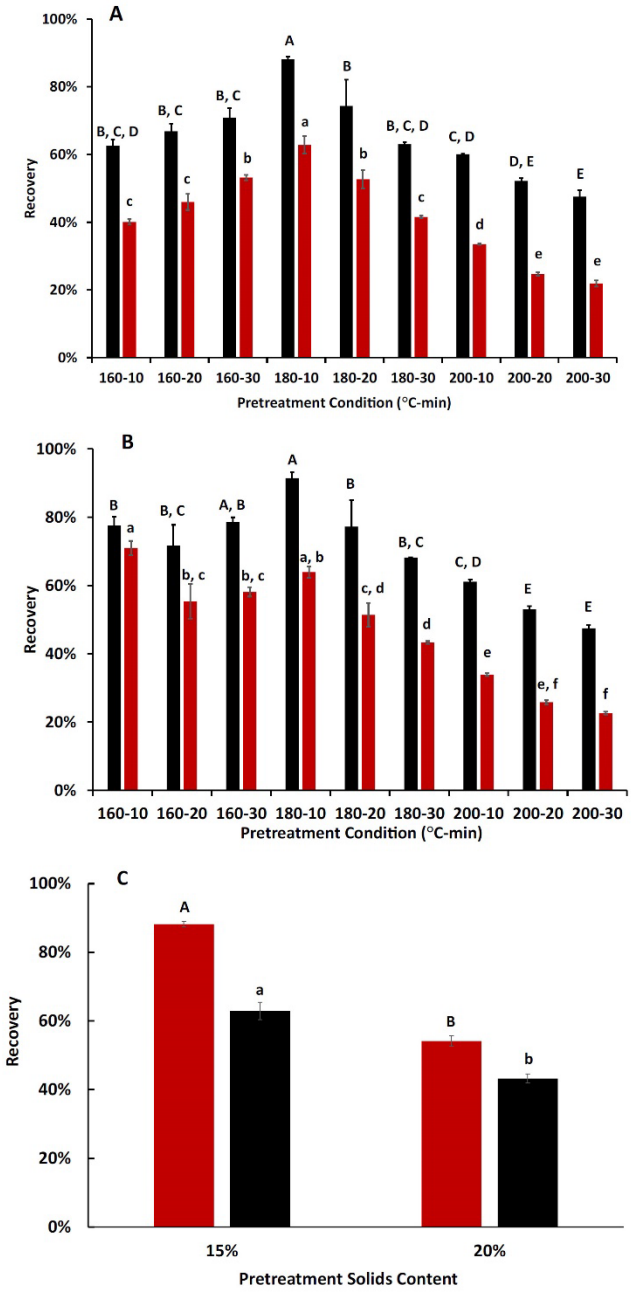

Figure 1. Glucose (red) and total sugar (black) recovery for different pretreatment conditions after pretreatment (PTR) (A) and after pretreatment and enzymatic hydrolysis (PHYR) (B) of corn fiber. Part C shows PHYR after pretreatment of CF at 180°C for 10 min with 15% or 20% solids. Different capital letters represent significant differences for glucose recoveries among pretreatment conditions ($p < 0.05$), and different lowercase letters represent significant differences for total sugar recoveries among pretreatment conditions ($p < 0.05$).

Enzymatic hydrolysis at 5, 10, and 15% solids loading were conducted for corn fiber pretreated at 15% solids, 180°C, 10 min. Glucan to glucose yields were similar at 5, 10, and 15% solids loading reaching 87.9, 90.2, and 93.3%, respectively. Xylan to xylose yields was 39.6% at 15% solids loading and higher than at 10 (36.9%) and 5% (33.2%) solids loading. Glucose concentration was highest at 15% solids loading reaching 36.3 g/L compared to 10 and 5% solids with 23.4 and 11.4 g/L, respectively (Fig. 2). With high glucan to glucose and xylan to xylose recoveries and high sugar concentrations, solids loading of 15% was chosen for enzymatic hydrolysis for corn fiber pretreated at 180°C, 10 min.

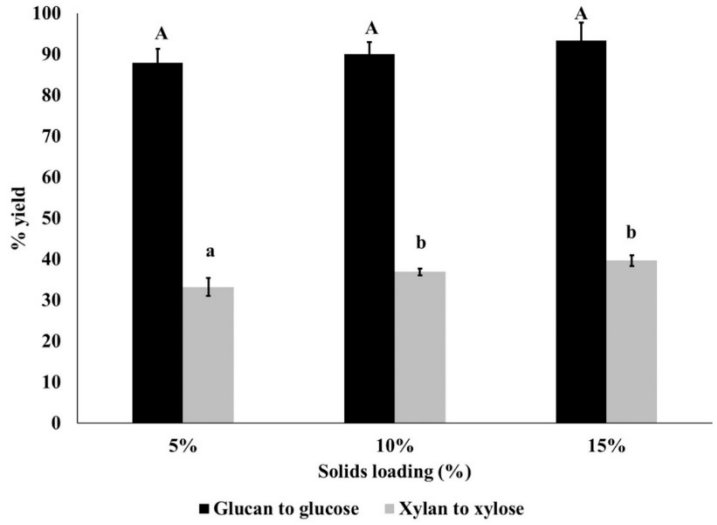

Figure 2. Glucose and xylose concentrations after enzymatic hydrolysis at 5, 10, and 15% solids loading. CF pretreated at 180°C-10 min. Different capital letters represent significant differences of glucose among solids loading groups ($p < 0.05$), and different lowercase letters represent significant differences of xylose among solids loading groups ($p < 0.05$).

3.3. Succinic acid production from control 1, CFHW, and CFHP

To study the feasibility of using CF hydrolysates as a low-cost, renewable source for SA production, fermentation of CF hydrolysates and controls that mimic the sugar contents in the CF hydrolysates with *A. succinogenes* were carried out. Figure 3 shows SA production from the CF hydrolysates and their controls. Glucose, xylose, and arabinose (the main sugars in CF) were simultaneously consumed during SA fermentation. Besides SA, formic acid (FA) and acetic acid (AA) also were produced during fermentation. It is noteworthy to mention that in both fermentations, SA, and by-products production as well as sugars concentrations seemed to remain constant after 36 h of fermentation. As shown in Figure 3A, the final SA concentration was 18.9 g/L from CFHW, while the final SA concentration from control 1 was 20.2 g/L. After 48 h of fermentation, 14.7% of initial glucose remained in control 1 and 32.4% of initial glucose remained in CFHW. The residual xylose concentrations in both CFHW and control 1 were 0.82 and 1.0 g/L, respectively.

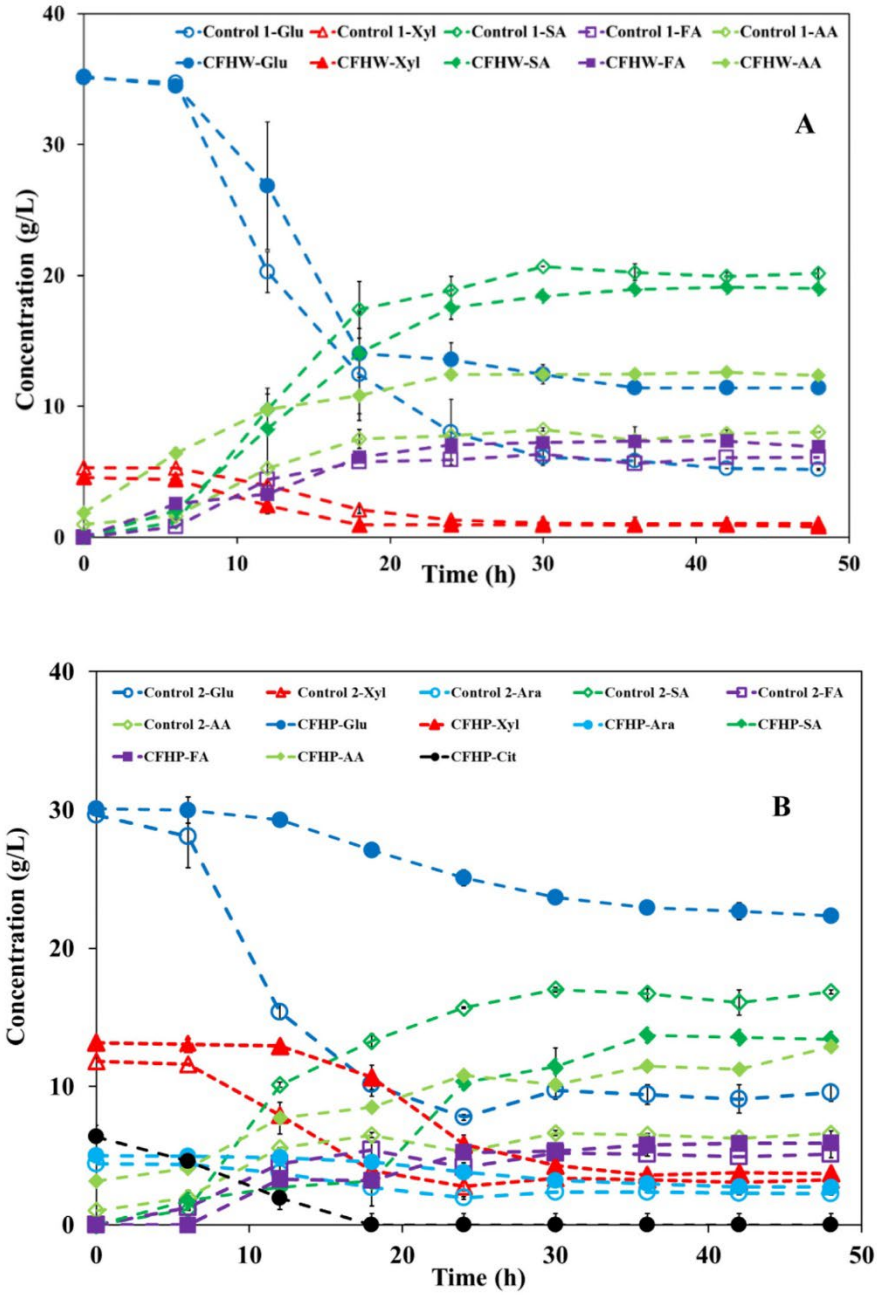

Figure 3. Time course of SA fermentation from A: Control 1 and CFHW (Initial glucose and xylose concentrations of 35.2 g/L and 4.5 g/L, respectively) and B: control 2 and CFHP (Initial glucose and xylose concentrations of 30 g/L and 12.5 g/L, respectively). Data are average values of duplicate experiments, and error bars represent compound standard deviation. Glu: glucose, Xyl: xylose, SA: succinic acid, FA: formic acid, AA: acetic acid, and Cit: sodium citrate.

As shown in Figure 3B, a final SA concentration of 13.7 g/L from CFHP was obtained compared to a final SA concentration of 16.7 g/L from control 2. After 48 h of fermentation, 32.2% and 74.2% of initial glucose were still present in control 2 and CFHP, respectively. The consumption of glucose in CFHP was inhibited during fermentation. The inhibitory environment could be attributed to the presence of furfural that was produced during LHW pretreatment. Analysis of the prehydrolysate showed furfural concentrations of 3.7 g/L (data not shown here).

Figure 3 also shows that SA and AA were produced with very little sugars consumption after 6 h of fermentation in CF hydrolysates. For CFHW, 2.05 g/L SA, 2.55 g/L, and 4.57 g/L AA were produced; however, only about 2% glucose and 4% xylose were consumed by the cells. In the case of CFHP, 1.77 g/L SA, and 0.93 g/L AA were produced; nevertheless, only about 0.8% of the sugars were consumed. Sodium citrate was used as a buffer in the enzymatic hydrolysis step, which led to citrate-rich CF hydrolysates. Figure 3B shows that sodium citrate was consumed instead of glucose and xylose in the first 18 h of fermentation. To determine what products were produced from citrate metabolism, a fermentation was performed using citrate as the sole carbon source and the results of this fermentation are shown in Figure 4. Figure 4 shows that *A. succinogenes* metabolized citrate to produce principally AA. SA and FA were also produced from citrate, while no LA and ethanol accumulation were observed during fermentation. It has been found that *A. succinogenes* lacks a complete TCA cycle. Two prior studies reported the absence of citrate synthase and isocitrate dehydrogenase enzymes activity in either anaerobically or aerobically grown *A. succinogenes* (McKinlay et al., 2010, 2005). McKinlay et al. (2005) pointed out that *A. succinogenes* was not capable of growth when citrate or isocitrate was supplied with NH_4Cl or aspartate. However, the findings in this paper differ from what was reported previously (McKinlay et al., 2010, 2005). These results could be attributed to the fact that *A. succinogenes* does have a citrate lyase (Asuc_0305, 1194-6 and 1198) (McKinlay et al., 2010), which also agrees with the findings of Van Der Werf et al. (1997), who reported citrate lyase activity in *A. succinogenes* cell extracts. These findings provide insight into SA biosynthesis by *A. succinogenes*. To perform an accurate measurement of the metabolites produced from CF hydrolysate by *A. succinogenes*, sodium acetate buffer (pH = 5.0, 50 mM) was used to produce CF hydrolysates for all subsequent experiments.

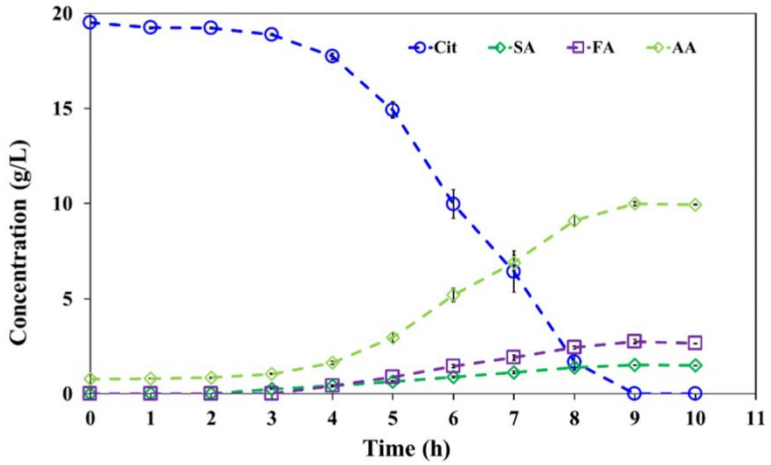

Figure 4. Time course of SA fermentation from citrate. Data are mean values of duplicate experiments, and error bars represent one standard deviation. Cit: sodium citrate, SA: succinic acid, FA: formic acid, and AA: acetic acid. The experiment was repeated twice, and the data points are the mean of two replicates.

3.4. Effect of a pH regulator ($MgCO_3$) on SA fermentation

Previous studies have reported that cell growth and SA production are affected by the level of CO_2 and pH during fermentation (Liu et al., 2008; Wang et al., 2012). CO_2 is a co-substrate in the production of SA and influences SA productivity and catabolite distribution in *A. succinogenes*. In general, high CO_2 concentration could increase the ratio of SA to by-products, leading to an improved SA yield. Literature reports that solid $MgCO_3$ concentration at 80% of initial glucose concentration has been supplied in the fermentation medium to buffer the pH and supply CO_2 during SA fermentation (Chen et al., 2011; Liu et al., 2008). Therefore, the effect of $MgCO_3$ to initial glucose concentrations ratio (0.8:1 and 1:1) on SA acid fermentation was investigated using CFHW and Control 1. When the Mg:glucose (Mg:Glu) ratio was 1:1, sugars were rapidly consumed by 36 h of fermentation and a maximum SA concentration from CFHW and Control 1 was achieved. Glucose, xylose, SA, other organic acids, and ethanol profiles during 36 h of fermentation of Control 1 and CFHW are displayed in Figure 5.

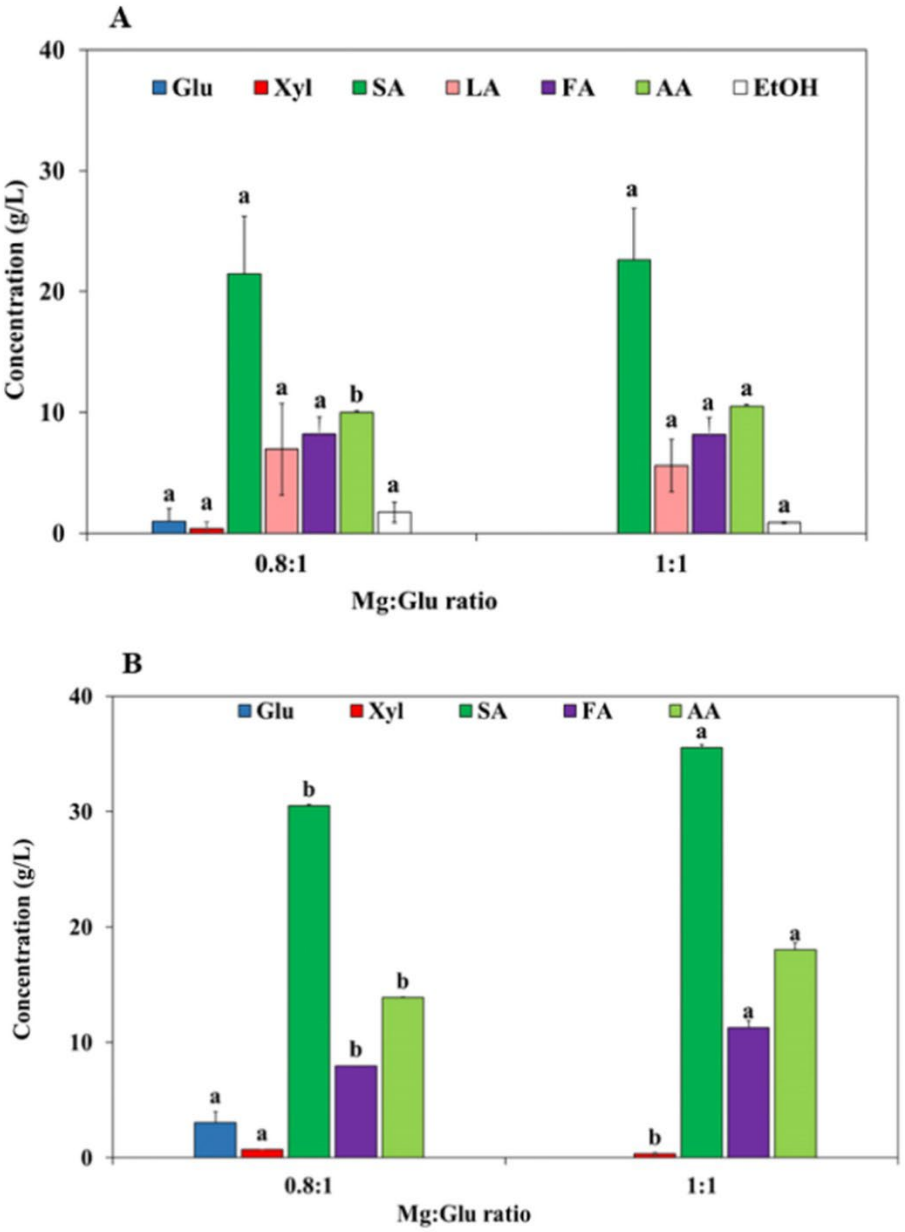

Figure 5. Effect of $MgCO_3$:glucose (Mg:Glu) ratio in SA and by-products concentrations at 36 h of fermentation (Initial glucose and xylose concentrations of 47 g/L and 4.7 g/L, respectively). A. Control 1 B. CFHW. Glu: glucose, Xyl: xylose, SA: succinic acid, LA: lactic acid, FA: formic acid, AA: acetic acid, and EtOH: ethanol. Data are average values of duplicate experiments, and error bars represent compound standard deviation. Different lowercase letters represent significant differences among the Mg:Glu ratio groups for the same compound ($p < 0.05$).

Figure 5A shows that 36 h SA concentrations in Control 1 were 21.5 g/L with a Mg:Glu ratio of 0.8:1 and 22.6 g/L with a Mg:Glu ratio of 1:1; however, there was no significant difference between these two concentrations. In addition to FA and AA, LA and ethanol also were produced during fermentation. Figure 5B shows that 36 h SA concentrations in CFHW were 30.5 g/L with a Mg:Glu ratio of 0.8:1 and 35.5 g/L with a Mg:Glu ratio of 1:1. SA concentration increased significantly when the Mg:Glu ratio was increased from 0.8:1 to 1:1. FA and AA were also detected during the fermentation, but no LA or ethanol was produced during fermentation of CFHW. Also, no glucose was present in both fermentations (control 1 and CFHW) at the 1:1 ratio, but glucose was still present at 0.8:1. A lack of CO_2 with a Mg:Glu ratio of 0.8:1 resulted in reduced glucose consumption, which would explain the residual glucose observed in the fermentations described in Section 3.3.

SA concentrations from CFHW were 29.5% (Mg:Glu ratio = 0.8:1) and 36.3% (Mg:Glu ratio = 1:1) greater than the SA concentrations from control 1 despite both media having equal sugar concentrations. Enzymatic hydrolysis of CF using sodium acetate buffer led to acetate rich CFHs and this is one of the major differences between the sugar control and the CFH. The findings here suggest that not only the level of CO_2 influences on SA formation, but there is also a pH threshold required in favor of SA production with respect to other end products, such as LA and ethanol. As reported in Liu et al. (2008), the optimal pH for SA productivity from glucose by *A. succinogenes* was 6.0–7.2, with a maximum production of SA when culture pH was maintained at 6.7. In addition, the authors stated that LA was produced during the fermentation in fed-batch cultivation mode. However, they neither reported the LA concentrations produced nor discussed the influence of pH on LA production.

The production of LA and ethanol in unbuffered media is not totally understood. It could be hypothesized that acetate buffer in the CFHW will establish the conditions to enhance the carbon flux to SA, compare to media without buffer that will possess the cultivation parameters to produce SA, but also to enhance the metabolic pathway of *A. succinogenes* toward lactate and ethanol production. To the authors' knowledge, no previous studies have shown how the utilization of buffer solutions influences SA fermentation, specifically on flask batch production for which pH is uncontrolled.

3.5. Effect of buffer solution capacity on SA fermentation

To explore the influence of buffer in the SA fermentation, sodium acetate buffer was added to the control in the same amount as in CFHW and SA; other organic acids concentrations were measured as well. As shown in Figure 6, the use of acetate buffer had a strong impact on succinate fermentation. When acetate buffer was added to the control (Control 3), the SA concentration was 28.7 g/L (yield of 0.67 g SA/g sugars); whereas SA concentration was 26.1 g/L (0.52 g SA/g sugars) in Control 1, which had no buffer (Fig. 6A). SA concentration was 27.8 g/L SA (0.61 g SA/g sugars) in CFHW. There was no significant difference between CFHS and control 3 SA yields ($p > 0.05$); however, SA yield from control 1 was significantly less than SA yields from control 3 and CFHW ($p > 0.05$). In fact, it could be observed that after 48 h of fermentation, 25.4% of initial glucose and 5.2% of initial xylose remained in Control 3; whereas 16.4% of initial glucose and 18.3% of initial xylose were present in CFHW and only 4.7% of initial glucose and 5.2% of initial xylose remained in Control 1.

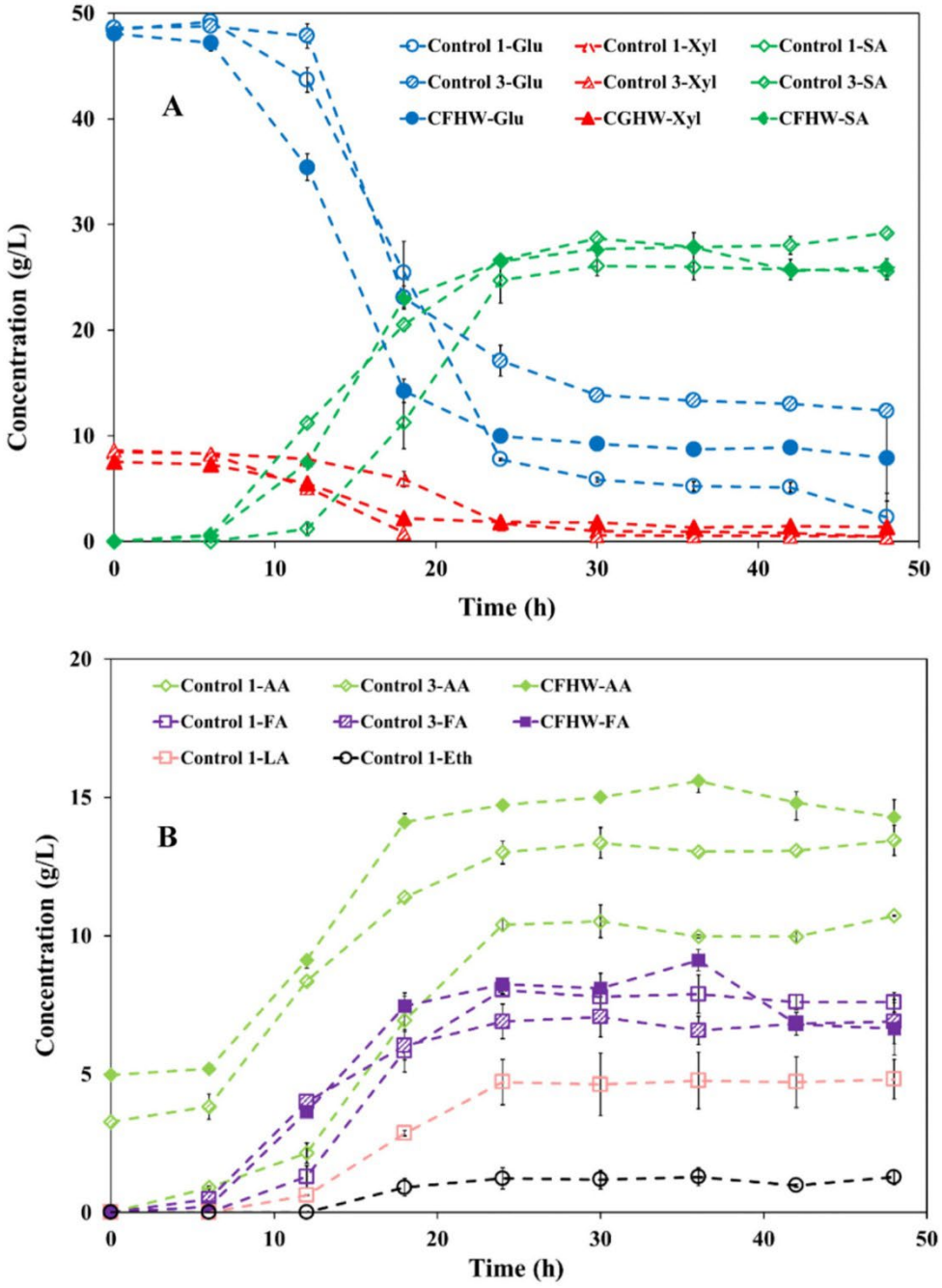

Figure 6. Time course of SA fermentation from control 1, control 3 and CFHW (Initial glucose and xylose concentrations of 48 g/L and 7.5 g/L, respectively). Data are average values of duplicate experiments, and error bars represent compound standard deviation. Variables shown in A are Glu: glucose, Xyl: xylose, and SA: succinic acid and variables shown in B are LA: lactic acid, FA: formic acid, AA: Acetic ACID, and EtOH: ethanol.

As shown in Figure 6B, AA and FA were the main by-products from CFHW and Control 3. From 6 to 24 h, the concentration of AA increased more rapidly than did other by-products. No significant increases of FA and AA were observed after 24 h. Conversely, ethanol and LA were present in detectable amounts in fermentation of Control 1 but not in fermentation of CFHW and Control 3. The findings here are contradictory to those reported by McKinlay et al. (2010) who stated that *A. succinogenes* does not produce lactate. They concluded that the single lactate dehydrogenase enzyme encoded in the *A. succinogenes*'s genome is involved with lactate oxidation to amino acids and sugar transport rather than lactate generation. In this study, LA was produced during fermentation of control 1. To date, three previous studies have reported LA formation in SA anaerobic production from wild-type *A. succinogenes* (Li et al., 2010; Liu et al., 2008; Wang et al., 2014). Li et al. (2010) reported that *A. succinogenes* can produce a high-titer and high-yield of LA in a dual-phase fermentation (aerobic and anaerobic cultivation) and that initial oxygen aeration of the fermentation enabled *A. succinogenes* to yield up to 1.93 mol LA/mol glucose compared to SA yield of 0.37 mol SA/mol glucose. Their study also revealed that lactate dehydrogenase activity in dual-phase fermentation was nearly 18-fold higher than values in a mono-phase process (anaerobic cultivation). Wang et al. (2014) used a microfiltration membrane to recover LA production by *A. succinogenes*, also in a dual-phase fermentation system. As previously described, Liu et al. (2008) observed LA formation in SA fermentation; however, they did not report the LA concentrations achieved.

Results in this investigation suggest that succinate versus lactate production from sugars present in CF hydrolysate by *A. succinogenes* are regulated by CO_2 and culture pH. The production of LA by *A. succinogenes* and its relationship with pH has not been previously reported. In this study, media pH was measured prior to batch fermentation. Initial media pH was 8.7, 8.0, and 7.9, in Control 1, CFHW, and Control 3 respectively, with a gradual pH decline to 6.0, 5.8, and 5.7 in Control 1, CFHW, and Control 3, respectively after 48 h of fermentation (data not shown here). High initial media pH favored lactate production later in the fermentation. In this regard, Samuelov et al. (1991) reported that low pH resulted in increase of the activity of the PEP carboxykinase enzyme, leading to an increase toward SA production. In their study, the authors concluded that SA production was induced by low pH and sufficient CO_2 availability and LA production was induced by high pH. At high pH (7.2) and insufficient CO_2 conditions, lactate dehydrogenase and ethanol dehydrogenase (enzymes responsible for production of lactate and ethanol, respectively) activities were detected, and PEP carboxykinase enzyme activity was lower. In contrast, at low pH (6.2) and sufficient CO_2 supply, succinate accumulates as a major product, and lactate dehydrogenase and alcohol dehydrogenase activities were insignificant, whereas the PEP carboxykinase activity was high. Even though the microbe used in their study was different (*Anaerobiospirillum succiniciproducens*, another SA producer), it can be speculated that a similar behavior occurs in the fermentations with *A. succinogenes*, since in both microorganisms glycolysis and the oxidative pentose phosphate pathway metabolized glucose to PEP and then to oxaloacetate to produce SA.

4. Conclusions

CF hydrolysate produced by optimized LHW pretreatment and enzymatic hydrolysis conditions supported SA production. SA concentration of 27.8 g/L with a yield of 0.61 g SA/g sugars was obtained when CF hydrolysate sugars were fermented by *A. succinogenes*. Consumption of citrate in buffer used to control pH during CF hydrolysis resulted in acetate production and reduced SA production. In addition, considerations associated with LA and ethanol production and its relationship with the pH control could be overcome by using systems where the pH can be controlled over time.

Authors' contribution statement – *Lisbeth Vallecilla-Yepez:* conceptualization, investigation, writing—original draft. *Divya Ramchandran:* conceptualization, investigation, writing—original draft. *Dianna Long:* investigation, writing—review and editing. *Rajib Saha:* conceptualization, resources, funding acquisition, supervision, writing—review and editing. *Mark R. Wilkins:* conceptualization, resources, funding acquisition, supervision, writing—review and editing.

Declaration of competing interest – The authors declare they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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